CHAPTER 16. AGAROSE THIN-LAYER ISOELECTRIC FOCUSING (TLIEF) FOR SPECIES DETERMINATION OF RAW MUSCLE TISSUES

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16.1 Introduction

Improvements in the developed biochemical technique of isoelectric focusing have allowed the application of this technique to be used for species determination of raw muscle tissue. This method provides for the relatively rapid species determination of a large number of samples in a definitive, less subjective manner, in a single analytical run without the use of anti-species sera. The principle of this technique involves the separation and focusing of proteins under an electrical field in a stable pH gradient dependent upon differences in the isoelectric points of the various proteins present. Since various species tissues contain multiple proteins of different isoelectric points, an aqueous extract of a particular species tissue when subjected to TLIEF will produce a stained protein band pattern unique and distinct for that species. By using the method described below, a total of 24 samples (48 if sample filter papers are cut in half along their long axis) may be analyzed in a single determination in one day as to their correct species. The use of this established method is intended to aid in the rapid species analysis of a large influx of raw tissue samples resulting from particular meat species problems which may be encountered in the Agency's inspection system.

16.2 Materials and Equipment

a. Multiphor for high Performance Analytical Electrofocusing in Agarose; to include 2117-301 Multiphor Basic Unit, 2117-107 Analytical Electrofocusing Lid, 2117-701 Capillary Gel Casting Kit, and 1850-100 Agarose-EF Accessory Kit. (LKB Instruments.)
b. 2197-001 D.C. Power Supply for Electrofocusing and Electrophoresis. (LKB Instruments.)
c. 185-101 Multiphor Gelbond film, 124 x 258 mm. (LKB Instruments.)
d. 2030-710 Bayonet female plastic tubing connector and 2030-702 Bayonet male plastic tubing connector. (LKB Instruments.)
e. 2117-109 Multiphor Staining Kit. (LKB Instruments.)
f. 1403 Coomassie Brilliant Blue R-250 dye (Fisher).
g. S-460 D-sorbitol powder, reagent grade (Fisher).

h. A-322 Trichloroacetic acid, reagent grade (Fisher).
i. A-297 5-sulfosalicylic acid, crystal, reagent grade (Fisher).
j. 14-198-5A High pressure hose clamps, 1/4" to 5/8" size (Fisher).
k. K-10 Kerosene (Fisher).
l. 17-0468-01 Agarose IEF (Pharmacia Fine Chemicals).
m. 17-0453-01 Pharmalyte Carrier Ampholyte, pH 5-8 range (Pharmacia).
n. Schleicher and Schuell #470 filter paper, 12.5 x 26 cm size and Schleicher and Schuell #577 filter paper, 12.5 x 26 cm size (PGC Scientific Corp.).
o. W 3237-10 Lauda Brinkman, Model K-4/RD Circulating water bath. (American Scientific Products.)
p. B-1206-2 Whirl-Pak® bags, 3" x 5". (American Scientific Products.)
q. R5316-8 Tygon tubing, formula S-50-HL, 5/16" x 1/16". (American Scientific Products.)
r. Hair dryer (hot and cold).
s. Rubber print roller, 6" wide.
t. Silicone gasket, 0.75 mm thick, overall dimensions of 12.5 x 26 cm, 3 sided of 5 mm width. (Potomac Rubber Co., Inc., Washington, DC.)
u. Water bath and incubator/oven capable of maintaining 65°C.
v. Centrifuge capable of 9,000 x G maximum.
w. Stomacher®

16.3 Procedure

a. Initial Reagent Preparations

i. Fixing solution:

Dissolve 25 g sulfosalicylic acid and 50 g of trichloroacetic acid in distilled water and dilute to a final volume of 500 ml.

ii. Destaining solution:

Mix 700 ml of ethyl alcohol and 200 ml glacial acetic acid together and dilute to a final volume of 2,000 ml with distilled water.

iii. Staining solution:

Completely dissolve 1 g Coomassie Brilliant Blue R250 dye in 500 ml of destaining solution.

iv. Cathode solution: (1 M NaOH, 100 ml)

v. Anode solution: (0.05 M H_2SO_4, 100 ml)

b. Sample Preparation

i. Obtain 1 g of diced, raw, muscle tissue and place in a small whirl-pak® bag together with 9 ml of distilled water.

ii. Thoroughly macerate the tissue by stomaching for 1-2 minutes and then leave overnight at 4°C.

iii. Centrifuge the resulting solution at 9000 x g for 10 minutes at room temperature and apply to sample filter papers when ready to electrofocus.

c. Apparatus Assembly

i. Set up and align the Lauda K-4/RD circulating water bath, LKB 2117 Multiphor Basic unit, and LKB 2197 D.C. Power supply on a laboratory bench such that the water bath is adjacent and convenient to the Multiphor unit and the power supply is on the adjacent side of the Multiphor unit.

ii. When placed on the same table or workbench, the LK-4/RD circulating waterbath causes a vibration problem that may cause the bands on the final agarose gel plate to be irregular. This problem can be corrected by isolating the waterbath, either by moving the waterbath to a separate table or to the floor. In cases where the lab has a raised or suspended floor, the addition of vibration damping elements (Fisher 01-914045) may be necessary to further isolate the vibration.

iii. Install the cooling plate in the Multiphor unit according to LKB instruction manual and attach appropriate, insulated, circulation hoses to the water bath and secure to make leak-proof.

iv. Adjust and calibrate the water bath temperature to assure an adequate supply of water is circulating through the cooling plate at 4°C.

v. Turn on the circulating, calibrated water bath at least 30 minutes prior to the preparation of a gel plate on the day that an analytical run is to be performed.

d. Agarose Gel-plastic Film Preparation

i. Mix 0.3 g Agarose-IEF (Pharmacia) and 3.6 g sorbitol in a conical flask with 27 ml distilled water and heat with stirring in a boiling water bath until all solids are dissolved.

ii. Place the flask containing the dissolved ingredients in a 65°C water bath and allow the solution to cool and equilibrate to 65°C.

iii. Add 1.9 ml of Pharmalyte, pH 5-8 range, ampholyte solution (Pharmacia) with needle and syringe, while gently swirling the 65°C tempered, liquid agarose solution. The final agarose solution is 30 ml total volume with an ampholine concentration of about 2.5% and agarose concentration of 1%. Leave the liquified agarose solution in the 65°C water bath until needed, after completing step (viii).

iv. Obtain a glass plate 125 x 260 mm (LKB 2117-701) Capillary Gel Casting Kit) that has been previously treated with the surface wetting agent Prosil-28 according to product instructions and place a small amount of distilled water on the glass surface.

v. Obtain a sheet of gel-bond film and place it on the wet glass plate such that the hydrophobic side of the sheet is down and in contact with the water and the hydrophilic side is up. Properly align the edges of the film sheet with the edges of the glass plate and remove excess water and air bubbles by rolling the surface of the film sheet with a rubber roller. Carefully remove excess water with absorbent towels.

vi. Place the three-sided, orange, silicone gasket on the film sheet and align the gasket edges with the edges of the film sheet.

vii. Place a 125 x 260 mm Prosil-28 treated glass plate on top of the orange gasket and align the leading edges with the gasket. Place five clamps around the three gasket-glass edges (2 each on long sides and 1 on the short end). When properly set up you will have a glass-film sheet sandwich arrangement which is leak proof on three sides where the gasket is and one open end with a space of about 0.75 mm (equal to gasket thickness) between the bottom of the top glass plate and the top of the gel-bond film sheet.

viii. Place this glass-film sheet sandwich arrangement in a 60-65°C oven for 10 minutes to warm up along with a 50 cc syringe and 21 gauge needle.

ix. Remove the warm glass-film sheet sandwich from the oven and set-up on a rack near the water bath containing the previously prepared liquid agarose solution at 65°C. Quickly fill a 50 cc syringe fitted with a 1 inch 21 gauge needle with the liquid agarose solution. Insert the needle in the space between the gel bond film sheet and bottom of the top glass plate. Rapidly but evenly inject the liquid agarose solution to fill this space without air bubbles before the agarose solution starts to gel.

x. Allow the agarose filled sandwich to set undisturbed until the agarose has solidified and then place in a refrigerator for 30 minutes to completely solidify the agarose.

xi. Carefully remove the five clamps and the top glass plate from the sandwich and obtain the agarose coated gel-bond film sheet from the bottom glass plate. When properly executed you will have a gel-bond film sheet containing a uniform, bubble free solidified agarose-ampholine layer of approximately 0.75 mm thickness.

xii. Several agarose gel-bond film plates may be prepared at the same time in order to reduce preparation time for future runs. The prepared plates must be preserved until needed by storage in the LKB Humidity Chamber (LKB-2117-110). These chambers are stackable and come in a kit holding up to three gel plates. Plates stored refrigerated for as long as 6 weeks in the humidity chamber show no loss in performance.

NOTE: Do not perform step (xi) above until just prior to starting step (iii) of section (e) below.

e. Isoelectric Focusing of Samples and References

i. Smear a small amount of reagent grade kerosene (Fisher) on the top of the cooling plate (which has 4°C water circulating through it) of the Multiphor unit.

ii. Place an LKB sample position template on top of the kerosene covered cooling plate, position in proper alignment with the cooling plate and smooth out so that no air bubbles are present under the template. Blot excess kerosene from edges of the template with absorbent towels.

iii. Smear a small amount of kerosene on top of the template and place the previously prepared agarose film sheet on top of the kerosene covered template, align edges with the cooling plate, remove any trapped air bubbles and blot excess kerosene from the edges.

iv. Soak filter paper strips (10 x 5 mm) in sample or reference tissue extracts and apply to the surface of the agarose gel near the anode using the visible template under the agarose-film sheet as a guide. A maximum of 24 samples total (including desired reference extracts) may be placed on the agarose surface. Be sure that the sample paper strip is in complete contact with the agarose surface and rinse off the tweezers between the handling of each sample strip with distilled water.

An alternative approach to sample application is to first place 24 blank paper strips in the proper position on the agarose surface and then with the use of a micropipetting device place a standard amount (25 µl) of sample extract on each respective strip. If it is desirable to employ small paper strips (10 x 2.5 mm) to accommodate a larger number of samples (48) for analysis, these strips should have only 10-15 µl sample extract applied to them and care must be taken to not cause overloading and mixing of adjacent samples.

v. Soak electrode filter paper strips with appropriate solutions for cathode (1 M NaOH) and anode (0.05 M H_2SO_4), blot excess off on paper toweling and guided by the visible template apply the wet electrode strips to the surface of the agarose in the proper anode and cathode positions and cut to the proper size of the agar.

vi. Place the LKB electrofocusing lid on the Multiphor unit over the cooling plate in the proper alignment such that the platinum electrode wires are centered and make good firm, complete contact with the respective soaked anode and cathode filter paper strips.

vii. Connect the electrical cables of the electrofocusing lid to the small pins on the front of the Multiphor unit.

viii. Mount the cover by first introducing the hooks on the cover into the rectangular holes on the rear side of the Multiphor unit, lower the cover and press the large electrode pins into the holes on the cover.

ix. Connect the electrical leads from the cover to the proper terminals (check for like charge) on the LKB 2197 D.C. power supply.

x. Turn on the power supply and adjust to provide the following conditions: 10 watts constant power, 700 V constant voltage and current unlimited (wide open) for a period of 45 minutes.

xi. After this period of time, change power to the following conditions: 10 watts constant power, 1000 V constant voltage and current unlimited for a period of 60 minutes.

xii. Turn off power after this period of time, remove the cover and electrofocusing lid and proceed to section (f) below.

f. Fixing, Staining, and Destaining

i. After completing the isoelectric phase of separation in Section 16.3 e, remove the agarose-film sheet, discard the electrode filter paper strips and sample filter paper strips. Place the agarose-film sheet in the LKB staining tray and immerse in fixing solution for 30 minutes with occasional gentle agitation. Perform this and all subsequent steps in a chemical fume hood with the exhaust turned on.

ii. Remove the agarose sheet from the first tray and place in a second tray containing destaining solution. Wash for a 30 minute period changing the fluid once.

iii. Remove the agarose sheet from the destaining solution and place on a glass plate. Place one sheet of Schleicher and Schuell #577 filter paper (12.5 x 26 cm) over the agarose surface so that no air pockets are trapped under the paper. Then place 2 sheets of Schleicher and Schuell #470 (12.5 x 26 cm) on top of the #577 filter paper, followed by a second glass plate and 1 kg weight. Allow sheets to remain in this manner for 15 minutes to effect an initial drying of the agarose gel.

iv. Remove the weight, glass plate, and filter papers (discard). Complete the thorough drying of the agarose gel with a draught of hot air from a hand held hair dryer. The agarose must be completely dry and adhering to the gel-bond sheet as a thin film of its' own before proceeding to the next step.

v. Place the dried agarose-film sheet in the staining solution for 10 minutes.

vi. Remove, drain, and place in destaining solution until background is sufficiently clear.

vii. Remove, drain, and dry to a final state with the hair dryer.

viii. Examine and compare the isoelectric focused protein patterns of the unknown samples to those of the reference tissue extracts used to identify the samples in question. The final dry preparation may be kept without further modifications as a permanent record of sample analysis.

16.4 Quality Control of Key Reagents or Procedures

In order to assure the integrity and reproducibility of the previously outlined TLIEF procedure, special attention should be given to the considerations cited below.

a. Agarose Gel-plastic Film Preparation.

Be sure to maintain the sterility of the stock ampholyte solution by using aseptic techniques and a new sterile needle and syringe to withdraw the necessary volume of ampholyte needed to prepare the liquified agarose solution. Ampholytes are susceptible to microbial contamination and this would destroy their intended function.

b. Do not allow air bubbles to form during the injection of the liquid agarose solution into the glass sandwich. Air bubbles at this stage will produce a void in that area on the solidified agarose sheet. The presence of air bubbles during electrofocusing will cause a discontinuous electrical resistance between the electrodes. This may ultimately result in improper band migration for the applied sample at that point.

c. Isoelectric Focusing of Samples and References.

Extracts from reference tissues should be prepared from relatively fresh tissues. Old tissues stored in the freezer for a period of time beyond 6-12 months begin to demonstrate fewer bands. Reference tissue extracts (controls) should be applied to each agarose sheet used for an analytical determination of unknown samples. Do

not rely on the use of previously prepared, dried, stained sheets of reference tissues for comparative purposes.

d. Fixing, Staining, and Destaining.

Proper staining contrast of the dried agarose sheet and protein bands depends upon complete removal of ampholytes and total drying of the agarose gel prior to staining.

Care should be given to wash well after the fixing step (step i; Section 16.3 f) and not to reuse the same quantity of fixing solution too many times as this will cause a build-up of ampholytes in it. Complete drying must be accomplished in step iv (Section 16.3 f) by careful use of the hot air dryer prior to staining (step v; Section 16.3 f). Destaining (step v; Section 16.3 f) must be accomplished carefully and empirically by frequent examination of the sheet to insure that under or over destaining is not allowed to occur such that all protein bands are optimumly stained and appear readily visible.

16.5 Selected References

Hamilton, W. D. 1982. Fish species identification by thin layer agarose isoelectric focusing and densitometric scanning. J. Assoc. Off. Anal. Chem. 65:119-122.

Pharmacia Fine Chemicals Agarose IEF pamphlet #52-1536-01.

Ukishima, Y., M. Kino, H. Kubota, S. Wada, and S. Okada. 1991. Identification of whale species by thin-layer isoelectric focusing of sarcoplasmic proteins. J. Assoc. Off. Anal. Chem. 74:943-950.